claim amendments is readily apparent from the teachings of the specification and the original claims.

Applicants have submitted the Sequence Listing **<u>from the parent application</u>** in both paper and computer readable form as required by 37 CFR 1.821(c) and (e). Amendments directing its entry into the specification have also been incorporated herein. The content of the paper and computer readable copies are the same. Further, the Sequence Listing contains the identical sequences appearing in the original application papers and, thus, no new matter has been added. In view of the Applicant's submission and the foregoing amendments, this application is now in compliance with 37 CFR 1.821-1.825.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"<u>Version with markings to show changes made.</u>"**

Applicants believe that the application is now in optimal form for examination. Such action is thus respectfully solicited.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

The claims have been amended as follows:

23. (Amended) [A] The Aspergillus mold fungus according to [Claim] claim 22, which [has been deprived of β-fructofuranosidase activity by deleting] is missing all or part of [the] a β-fructofuranosidase gene on [the] its chromosome DNA [of the original Aspergillus mold fungus].

24. (Amended) [A] The Aspergillus mold fungus according to [Claim] claim 23, which is an Aspergillus niger.

25. (Amended) [A] The Aspergillus mold fungus according to [Claim] claim 24, which is Aspergillus niger NIA1602 (FERM BP-5853).

26. (Amended) A process for producing a β-fructofuranosidase comprising:

transforming [a] the mold fungus according to [any one of Claims] claim 22, [to 25] using a DNA construction comprising a DNA encoding a β-fructofuranosidase,

cultivating the transformant, and

collecting the β-fructofuranosidase from the transformant and/or the culture thereof.

β-FRUCTOFURANOSIDASE AND ITS GENE, METHOD OF ISOLATING β-FRUCTOFURANOSIDASE GENE, SYSTEM FOR PRODUCING β-FRUCTOFURANOSIDASE, AND β-FRUCTOFURANOSIDASE VARIANT

This application is a Divisional application of Serial No. 09/142,623, filed September 10, 1998, now, allowed, which is a 371 of PCT/JP97/00757, filed March 11, 1997, which is pending.

Background of the Invention

1. Field of the Invention

The present invention relates to a β-fructofuranosidase gene, a process for isolating the gene, and a system for producing a β-fructofuranosidase. More particularly, the present invention relates to a novel β-fructofuranosidase, a DNA encoding it, and a process for isolating a DNA encoding β-fructofuranosidase; a novel mold fungus having no β-fructofuranosidase and a process for producing a recombinant β-fructofuranosidase using the mold fungus as a host; and a β-fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose.

2. ~~Background Art~~ Description of the Related Art

The molecular structure of a fructooligosaccharide is the same as that of sucrose, except that the fructose half of a fructooligosaccharide is coupled with another one to three fructose molecules at positions C1 and C2 via a β bond. Fructooligosaccharides are indigestible sugars known for their physiological advantages, such as the facilitation of Bifidobacterial growth in the intestines, metabolic stimulation for cholesterols and other lipids, and little cariosity.

Fructooligosaccharides are found in plants, such as asparagus, onion, Jerusalem-artichoke and honey. They are also synthesized from sucrose by the newly industrialized mass production technique using fructosyltransfer reaction which is catalyzed by a β-fructofuranosidase derived from a microorganism. However, as β-fructofuranosidase preparations which are currently used for the industrial production of fructooligosaccharides is a cell-bound β-fructofuranosidase derived from Aspergillus niger, They contain a relatively large proportion of proteins as impurities. Therefore, a need still exists for a high-purity β-fructofuranosidase preparation with little unwanted proteins and a high titer. Further, an

extracellular β-fructofuranosidase is desired in an attempt to improve efficiently by using it in a fixed form, as an extracellularly available enzyme is more suitable for fixation.

Genes encoding β-fructofuranosidase have been isolated from bacteria (Fouet, A., Gene, 45, 221-225 (1986), Martin, I. et al., Mol. Gen. Genet., 208, 177-184 (1987), Steininctz, M. et al., Mol. Gen. Genet., 191, 138-144 (1983), Scholle, R. et al., Gene, 80, 49-56 (1989), Aslanidis, C. et al., J. Bacteriol., 171, 6753-6763 (1989), Sato, Y. and Kuramitsu, H. K., Infect. Immun., 56, 1956-1960 (1989), Gunasekaran, P. et al., J. Bacteriol., 172, 6727-6735 (1990)); yeast (Taussing, R, and M. Carlson, Nucleic Acids Res., 11, 1943-1954 (1983), Laloux, O. et al., FEBS Lett., 289, 64-68 (1991); mold (Boddy, L. M. et al., Curr, Genet., 24, 60-66 (1993); and plants (Arai, M. et al., Plant Cell Physiol., 33, 245-252 (1992), Unger, C. et al. Plant Physiol., 104, 1351-1357 (1994), Elliott, K. et al., Plant Mol. Biol., 21, 515-524 (1993), Sturm, A. and Chrispeels, M. J., Plant Cell, 2, 1107-1119 (1990)). However, to the best knowledge of the inventors, no gene has been found which encodes a β-fructofuranosidase having transferase activity and is usable for the industrial production of fructooligosaccharides.

If a β-fructofuranosidase gene usable for the industrial production of fructooligosaccharides is obtained, other functionally similar genes may be isolated, making use of their homology to the former. To the best knowledge of the inventors, no case has been reported on the screening of a new β-fructofuranosidase gene using this technique. A process for isolating a β-fructofuranosidase gene by this approach may also be applied to the screening of β-fructofuranosidase enzyme to achieve significantly less effort and time than in conventional processes: first, using a β-fructofuranosidase gene as a probe, a similar β-fructofuranosidase gene is isolated, making use of its homology to the former; then, the isolated gene is introduced and expressed in a host which does not metabolize sucrose, such as Trichoderma viride, or a mutant yeast which lacks sucrose metabolizing capability (Oda, Y. and Ouchi, K., Appl. Environ. Microbiol., 1989, 55, 1742-1747); a homogeneous preparation of β-fructofuranosidase is thus obtained as a genetic product with significantly less effort and time of screening.

processing purpose while maintaining the general physiological advantages of fructooligosaccharides (Japanese Patent Application No. 222923/1995, Japanese Patent Laid-Open Publication No. 31160/1994). In this sense, they are fructooligosaccharide preparations having new features.

In consideration of the above, some of the inventors have proposed an industrial process for producing crystal 1-kestose from sucrose (Japanese Patent Application No. 64682/1996, Japanese Patent Application No. 77534/1996, and Japanese Patent Application No. 77539/1996). According to this process, a β-fructofuranosidase harboring fructosyltransferase activity is first allowed to act on sucrose to produce 1-kestose; the resultant 1-kestose is fractionated to a purity of 80% or higher by chromatographic separation; then, using this fraction as a crystallizing sample, crystal 1-kestose is obtained at a purity of 95% or higher. The β-fructofuranosidase harboring fructosyltransferase activity used in this process should be able to produce 1-kestose from sucrose at a high yield while minimizing the byproduct nystose, which inhibits the reactions in the above steps of chromatographic separation and crystallization. In the enzyme derived from Aspergillus niger, which is currently used for the industrial production of fructooligosaccharide mixtures, the 1-kestose yield from sucrose is approximately 44%, while 7% is turned to nystose (Japanese Patent Application No. 64682/1996). These figures suggest that the enzyme has room for improvement in view of the industrial production of crystal 1-kestose. As a next step, new enzymes having more favorable characteristics were successfully screened from Penicillium roqueforti and Scopulariopsis brevicaulis. These enzymes were able to turn 47% and 55% of sucrose into 1-kestose, respectively, and 7% and 4% to nystose (Japanese Patent Application No. 77534/1996, and Japanese Patent Application No. 77539/1996). Although these figures show that the new enzymes were superior to the enzyme derived from Aspergillus niger for higher 1-kestose yields and less nystose production from sucrose, the productivity and stability of the enzymes were yet to be improved. Thus, it is awaited to see a new enzyme that maintains the productivity and stability of the enzyme derived from Aspergillus niger,

which is currently used for the industrial production of fructooligosaccharide mixtures, while achieving a sucrose-to-1-kestose yield comparable or superior to that of the enzymes derived from Penicillium roqueforti and Scopulariopsis brevicaulis.

Summary of the Invention

The inventors have now successfully isolated a novel β-fructofuranosidase gene, and developed a process for isolating other β-fructofuranosidase genes using the novel gene.

The inventors have also successfully produced a novel mold fungus having no β-fructofuranosidase activity, and developed a system for producing a recombinant β-fructofuranosidase using the mold fungus as a host.

Further, the inventors have found that the characteristics of β-fructofuranosidase with fructosyltransferase activity change with its amino acid sequence, and have successfully produced a β-fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose.

The present invention is based on these findings.

Thus, the first aspect of the present invention provides a novel β-fructofuranosidase gene and a β-fructofuranosidase encoded by the gene.

The second aspect of the present invention provides a process for isolating a β-fructofuranosidase gene using the novel β-fructofuranosidase gene. The process according to the second aspect of the present invention also provides a novel β-fructofuranosidase.

In addition, the third aspect of the present invention provides a novel mold fungus having no β-fructofuranosidase activity and a system for producing a recombinant β-fructofuranosidase using the mold fungus as a host.

Further, the fourth aspect of the present invention provides a β-fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose.

Figure 2 shows expression vector pPRS01-Hyg in which a β-fructofuranosidase gene isolated in the process according to the second aspect of the present invention has been introduced.

Figure 3 is the restriction map of a DNA fragment comprising the niaD gene which has been derived from the Aspergillus niger NRRL4337.

Figure 4 shows the construction of plasmid pAN203.

Figure 5 shows the construction of plasmid pAN572.

Figure 6 is the restriction map of plasmid pAN120.

Figure 7 shows the construction of plasmid pY2831.

Figure 8 shows the construction of plasmid pYSUC (F170W).

Figure 9 shows the construction of plasmid pAN531.

Preferred Embodiments

~~Detailed Description of the Invention~~

Deposit of Microorganism

The novel mold fungus Aspergillus niger NIA1602 having no β-fructofuranosidase according to the present invention has been deposited in the National Institute of Bioscience and Human-Technology, Ministry of International Trade and Industry of Japan (Higashi 1-1-3, Tsukuba City, Ibaraki Pref., Japan) as of March 6, 1997, under Accession No. FERM-BP5853.

β-Fructofuranosidase according to the first aspect of the present invention

The polypeptide according to the first aspect of the present invention comprises the amino acid sequence of SEQ ID No. 1 as shown in the sequence listing. This polypeptide having the amino acid sequence of SEQ ID No. 1 has enzymatic activity as β-fructofuranosidase. The polypeptide according to the present invention involves a homologue of the amino acid sequence of SEQ ID No. 1 as shown in the sequence listing. The term "homologue" refers to an amino acid sequence in which one or more amino acids are inserted, substituted or deleted in, or added to either or both of the terminals of, the amino acid sequence of SEQ ID No. 1, while retaining β-fructofuranosidase activity. Such a homologue can be selected and produced by those skilled in the art without undue experiments by referring to the sequence of SEQ ID No. 1.

The β-fructofuranosidase having the amino acid sequence of

nucleotide sequence of SEQ ID No. 2, or a probe. Preferably, the probe should be marked.

The procedures for screening the gene library, marking the probe, isolating the marked and selected sequences, and further isolating a β-fructofuranosidase gene from the isolated sequences may be performed according to the standard techniques of genetic engineering under suitably selected conditions. Those skilled in the art would be able to select these procedures and conditions easily by referring to the sequence of SEQ ID No. 2.

On the other hand, process b) above comprises:

preparing a primer consisting of a nucleotide sequence which comprises all or part of the nucleotide sequence of SEQ ID No. 2 as shown in the sequence listing,

~~carring~~ carrying out PCR process on the primer using a sample which presumably contains a β-fructofuranosidase gene as a template, and

isolating a β-fructofuranosidase gene from the amplified PCR product.

The procedures for preparing the primer to be used, for preparing a sample which presumably contains a β-fructofuranosidase gene, and for PCR may be performed according to the standard techniques of genetic engineering under suitably selected conditions. Those skilled in the art would be able to select these procedures and conditions easily by referring to the sequence of SEQ ID No. 2.

The scope of application of the process for isolating a β-fructofuranosidase gene according to the present invention is not limited in any way provided that β-fructofuranosidase is presumably contained, such as Eumycetes, specifically Aspergillus, Penicillium or Scopulariopsis microorganisms.

Novel β-fructofuranosidase and gene encoding same obtained by the second aspect of the present invention

The process for isolating a gene according to the second aspect of the present invention provides a novel β-fructofuranosidase enzyme having the amino acid sequence of SEQ ID No. 11 or 13 as shown in the sequence listing.

The β-fructofuranosidase enzyme according to the present invention may be a homologue of the amino acid sequence of SEQ ID

marker gene as an index.

In the first step of hit-and-run substitution, a vector which bears a deactivated β-fructofuranosidase gene containing at least one mutation (preferably a deletion) which can independently deactivate the target β-fructofuranosidase gene and a selectable marker gene is prepared. This vector is then introduced in the cell to induce homologous recombination with the β-fructofuranosidase gene on the chromosome in the target β-fructofuranosidase gene on the upstream of the mutation. As a result, the vector bearing the selectable marker gene is now positioned between two copies of target β-fructofuranosidase gene on the chromosome — one with a mutation and one without. Next, the vector between the two copies of target β-fructofuranosidase gene is looped out, and allowed to homologously recombine again on the downstream of the mutation. As a result, the vector bearing the selectable marker gene and one copy of target β-fructofuranosidase gene is removed, leaving the target β-fructofuranosidase gene on the chromosome with a mutation. These recombinant strains can be selected with the absence of the marker gene as in index. It should be noted that the same effect is obviously achievable by inducing homologous recombination first on the downstream of the mutation, then on its upstream.

In the above procedures, any selectable marker gene may be used provided that a transformant is selectable. However, strains missing the selectable marker should be selected in the course of two-step gene targeting, it is preferable to use a selectable marker gene which allows these strains to be positively selected, such as nitrate reductase gene (niaD), orotidine-5'-phosphate decarboxylase gene (pyrG), or ATP sulfurylase gene (sC).

Examples of mold fungus according to the third aspect of the present invention include Aspergillus niger NIA1602 (FERM BP-5853).

Process for producing a recombinant β-fructofuranosidase using the mold fungus having no β-fructofuranosidase according to the third aspect of the present invention as a host

The mold fungus according to the present invention may preferably be used for producing recombinant β-fructofuranosidase.

More specifically, a DNA fragment encoding β-fructofuranosidase is introduced in the mold fungus according to the present invention in the form of a DNA molecule which is replicable in the host cell according to the present invention and can express the gene, particularly an expression vector, in order to transform the mold fungus. The transformant has then the ability to produce the recombinant β -fructofuranosidase and no other β-fructofuranosidase enzymes.

This procedure, where a preferred from of the DNA molecule is a plasmid, may be carried out according to the standard techniques of genetic engineering.

According to a preferred embodiment of the present invention, examples of DNA fragments encoding β-fructofuranosidase include the DNA encoding β-fructofuranosidase according to the first aspect of the present invention as described earlier, the DNA encoding a novel β-fructofuranosidase which has been isolated in the process according to the second aspect of the present invention, and the DNA encoding a β-fructofuranosidase variant according to the fourth aspect of the present invention as described later.

Examples of systems for expressing β-fructofuranosidase using the mold fungus according to the third aspect as a host include the expressing system which has been described in the first aspect of the present invention.

More specifically, it is preferable that the plasmid to be used bear a selectable marker gene for the transformant, such as a drug-resistance marker gene or marker gene complementing an auxotrophic mutation. Examples of preferred marker genes include hygromycin-resistance gene (hph), bialophos-resistance gene (Bar), nitrate reductase gene (niaD), orotidine-5'-phosphate decarboxylase gene (pyrG), and ATP-sulfurylase gene (sC).

It is also preferable that the DNA molecule for use as an expression vector contain nucleotide sequences necessary for the expression of the β-fructofuranosidase gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a translation termination signal, and a transcription termination signal. Examples of preferred promoters include, in addition to the promoter on the inserted fragment which is able to

acid residues at positions 170, 300, 313 and 386 in the amino acid sequence of SEQ ID No. 1 are substituted by other amino acids. The amino acids to be substituted in a homologue of the original β-fructofuranosidase consisting of the amino acid sequence of SEQ ID No. 1 are easily selected by comparing amino acid sequences by a known algorithm. If, however, comparison of amino acid sequences by a known algorithm is difficult, the amino acids to be substituted can be easily determined by comparing the stereochemical structures of the enzymes.

Preparation of a variant β-fructofuranosidase according to the fourth aspect of the present invention

The variant β-fructofuranosidase according to the fourth aspect of the present invention may be prepared by procedures such as genetic engineering or polypeptide synthesis.

When employing genetic engineering, the DNA encoding the original β-fructofuranosidase is first obtained. Next, mutation is induced at specific sites on the DNA to substitute their encoded amino acids. Then, an expression vector containing the mutant DNA is introduced in a host cell to transform it. The transformant cell is cultivated to prepare the desired β-fructofuranosidase variant.

Several methods are known to those skilled in the art for inducing mutation at specific sites on a gene, such as the gapped duplex method (Methods in Enzymology, 154, 350 (1987)) and the Kunkel method (Methods in Enzymology, 154, 367 (1987)). These methods are applicable for the purpose of inducing mutation at specific sites on a DNA encoding β-fructofuranosidase. The nucleotide sequence of the mutant DNA may be identified by procedures such as the chemical degradation method devised by Maxam and Gilbert (Methods in Enzymology, 65, 499 (1980)) or the dideoxynucleotide chain termination method (Gene, 19, 269 (1982)). The amino acid sequence of the β-fructofuranosidase variant can be decoded from the identified nucleotide sequence.

Production of a β-fructofuranosidase variant according to the fourth aspect of the present invention

The β-fructofuranosidase variant according to the fourth aspect of the present invention may be produced in a host cell by introducing a DNA fragment encoding β-fructofuranosidase in the

host cell in the form of a DNA molecule which is ~~replacatalbe~~ replicable in the host cell and can express the gene, particularly an expression vector, in order to transform the host cell.

Therefore, the present invention provides a DNA molecule, particularly an expression vector, which comprises a gene encoding the β-fructofuranosidase variant according to the present invention. The DNA molecule is obtained by introducing a DNA fragment encoding the β-fructofuranosidase variant according to the present invention in a vector molecule. According to a preferred embodiment of the present invention, the vector is a plasmid.

The DNA molecule according to the present invention may be prepared by the standard technique of genetic engineering.

The vector applicable in the present invention may be selected as appropriate, considering the type of the host cell used, from viruses, plasmids, cosmid vectors, etc. For example, a bacteriophage in the λ phage group or a plasmid in the pBR or pUC group may be used for E. coli host cells, a plasmid in the pUB group for Bacillus subtilis, and a vector in the YEp, YRp or YCp group for yeast.

It is preferable that the plasmid contain a selectable marker to ease the selection of the transformant, such as a drug-resistance marker or marker gene complementing an auxotrophic mutation. Preferred examples of marker genes include ampicillin-resistance gene, kanamycin-resistance gene, and tetracycline-resistance gene for bacterium host cells; N-(5'-phosphoribosyl)-anthranilate isomerase gene (TRP1), orotidine-5'-phosphate decarboxylase (URA3), and β-isopropylmalate dehydrogenase gene (LEU2) for yeast; and hygromycin-resistance gene (hph), bialophos-resistance gene (Bar), and nitrate reductase gene (niaD) for mold.

It is also preferable that the DNA molecule for use as an expression vector according to the present invention contain nucleotide sequences necessary for the expression of the β-fructofuranosidase gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a ribosome binding site, a translation termination signal, and a transcription termination signal.

Examples of preferred promoters include, in addition to the promoter on the inserted fragment which is able to function in the

host, promoters such as those of lactose operon (lac), and tryptophan operon (trp) for E. coli; promoters such as those of alcohol dehydrogenase gene (ADH), acid phosphatase gene (PHO), galactose regulated gene (GAL), and glyceraldehyde-3-phosphate dehydrogenase gene (GPD) for yeast; and promoters such as those of α-amylase gene (amy), glucoamylase gene (gla), cellobiohydrolase gene (CBHI), and β-fructofuranosidase gene for mold.

If the host cell is Bacillus subtilis, yeast or mold, it is also advantageous to use a secretion vector to allow it to extracellularly secrete recombinant β-fructofuranosidase. Any host cell with an established host-vector system may be used, preferably yeast, mold, etc. The use of a host cell without sucrose metabolizing capability would be particularly preferred, as it does not have an enzyme which acts on sucrose except the expressed β-fructofuranosidase variant and, therefore, allows the resultant β-fructofuranosidase variant to be used for the production of fructooligosaccharides without purification. Thus, according to a preferred embodiment of the present invention, the mold fungus according to the third aspect of the present invention may be used as the host cell. A few Trichoderma strains and a type of yeast may be used as the host without sucrose metabolizing capability (Oda, Y. and Ouchi, K., Appl. Environ. Microbiol., 55, 1742-1747, 1989).

Production of fructooligosaccharides using the β-fructofuranosidase variant according to the fourth aspect of the present invention

The present invention further provides a process for producing fructooligosaccharides using the β-fructofuranosidase variant. The process for producing fructooligosaccharides is practiced by bringing the host cell which synthesizes the β-fructofuranosidase variant, or the β-fructofuranosidase variant itself into contact with sucrose.

In the process using the β-fructofuranosidase variant, fructooligosaccharides may be produced and purified under substantially the same conditions as in the process for producing fructooligosaccharides using the β-fructofuranosidase according to the first aspect of the present invention.

Examples

The present invention will now be described in more detail using the following Examples. However, the Examples are merely illustrative in nature and should not be construed to limit the spirit and scope of the claims

Example A

ABSTRACT OF THE DISCLOSURE

A novel β-fructofuranosidase gene and a β-fructofuranosidase encoded by the gene, a process for isolating a β-fructofuranosidase gene using the novel β-fructofuranosidase gene, and a novel β-fructofuranosidase obtained by this isolation process are disclosed. A novel mold fungus having no β-fructofuranosidase activity suitable for the production of β-fructofuranosidase, and a system for producing a recombinant β-fructofuranosidase using the novel mold fungus as a host is disclosed. Further, a β-fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as 1-kestose from sucrose is disclosed.